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Identification of Small Molecule Inhibitors of microRNA Involved in Chemoresistance and Cancer Stem Cells for Ovarian Cancer Intervention

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14. ABSTRACT
During last year, we have screened NCI Diversity Set, Nature Compound and Clinical Collection 1 and 2 libraries using cell-based assay and have identified 22 and 35 candidate compounds that inhibit miR-19a/b and miR-221/222, respectively. Furthermore, we have characterized the specificity of these candidates as well as potential inhibitors of miR-214 and have identified 2 specific inhibitors for miR-214, 3 for miR-221/222 and 2 for miR-19a/b. We are currently investigating their anti-tumor activity in cell culture and animal model.

15. SUBJECT TERMS
microRNA, small molecule inhibitor, ovarian cancer, chemoresistance

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**Table of Contents**

Introduction

Three tasks have been proposed in this project: 1) Identify small molecule inhibitors of miR-19a/b and miR-221/222 using cell-based HTS assay 2) Determine the specificity of miR-19a/b, miR-214 and miR-221/222 inhibitors identified from Aim 1 and 3) Evaluate the small molecule inhibitors of miR-19a/b, miR-214 or miR-221/222 in overcoming ovarian cancer chemoresistance in cell culture and animal mode.

Body:

1. Identification of candidate compounds that inhibit miR-221/222 and miR-19a/b.

MiR-214 and miR-221/222 and are elevated in ovarian cancer, especially in chemoresistance and recurrent tumors (1-3). Our recent data showed that miR-214 and miR-19a/b positively regulated ovarian cancer stem cell property (4). Thus, we have aimed to identify small molecule inhibitors of these miRNAs by screening the NCI Diversity Set, Nature Compound and Clinical Collection 1 and 2 libraries using cell-based HTS assay. Briefly, we co-transfected OVCAR-422 cells in 96-well plate with pMIR-Luc-2x3'UTR/Bim and pMIR-Luc-2x3'UTR/p27 (e.g., miR-19a/b and miR-221/222 reporter, respectively) as well as pre-miR-19a/b and pre-miR-221/222. Following treatment with and without individual compound for 6 hours, luciferase activity was measured. Since pre-miR-19a/b and pre-miR-221/222 inhibit pMIR-Luc-2x3'UTR/Bim and pMIR-Luc-2x3'UTR/p27 activity, respectively, the compounds that overcome the effects of miR-19a/b and miR-221/222 on the luciferase activity will be considered as candidates of miR-19a/b and miR-221/222 inhibitors (Figure 1). The experiments were repeated three times in triplicate. Through these screening, we have identified 22 and 35 candidate compounds that inhibit miR-19a/b and miR-221/222, respectively (Figures 1 and 2).

A

```
    cuuugggUCGUCUGUUACAUCGa miR221          agucaaAACGUAU-CUAAACGUGu miR19a
          |||:     :||||||||                      || | |: | ||||||||
 5' cuCUAaaAGCGUUGGAUGUAGCa 3'p27         5' ccuggcUUACUUGUGUUUUGCACu 3' Bim
      |:|   |||  ::|||||||||                      || | || | ||||||||
    ugGGUCAUCG--GUCUACAUCGa miR222          agucaaAACGUAC-CUAAACGUGu miR-19b
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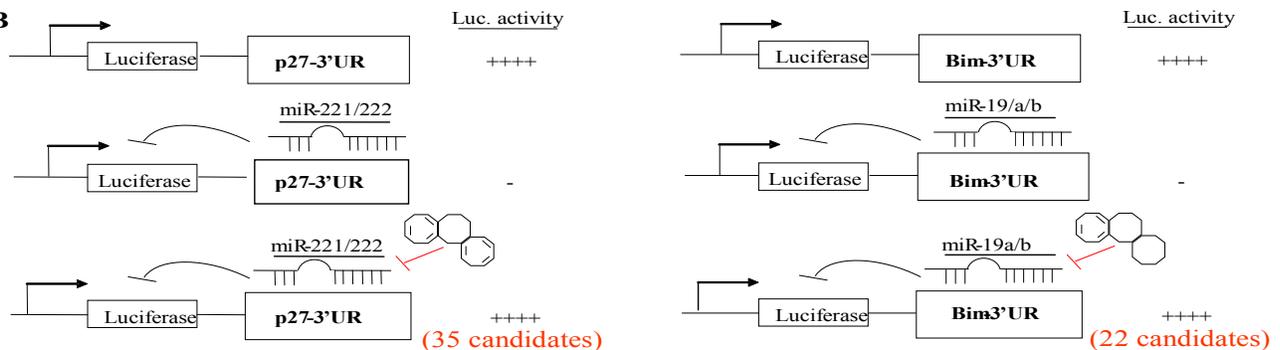

Figure 1. (**A**) Sequence alignment of miR221/222 with p27-3'UTR and miR19a/b with Bim-3'UTR (**B**) Diagram represents the cell-based luciferasescreening assay for small molecule inhibitors of miR221/222 (left) and miR19a/b (right).

4

Candidate: p2A6

Candidate: p3H6, p3G7, p3A10

Candidate: p5B2, p5E2, p5F7, p5H9, p5C11

Candidates: p6A2, p6B2, p6G3, p6F4, p6B6
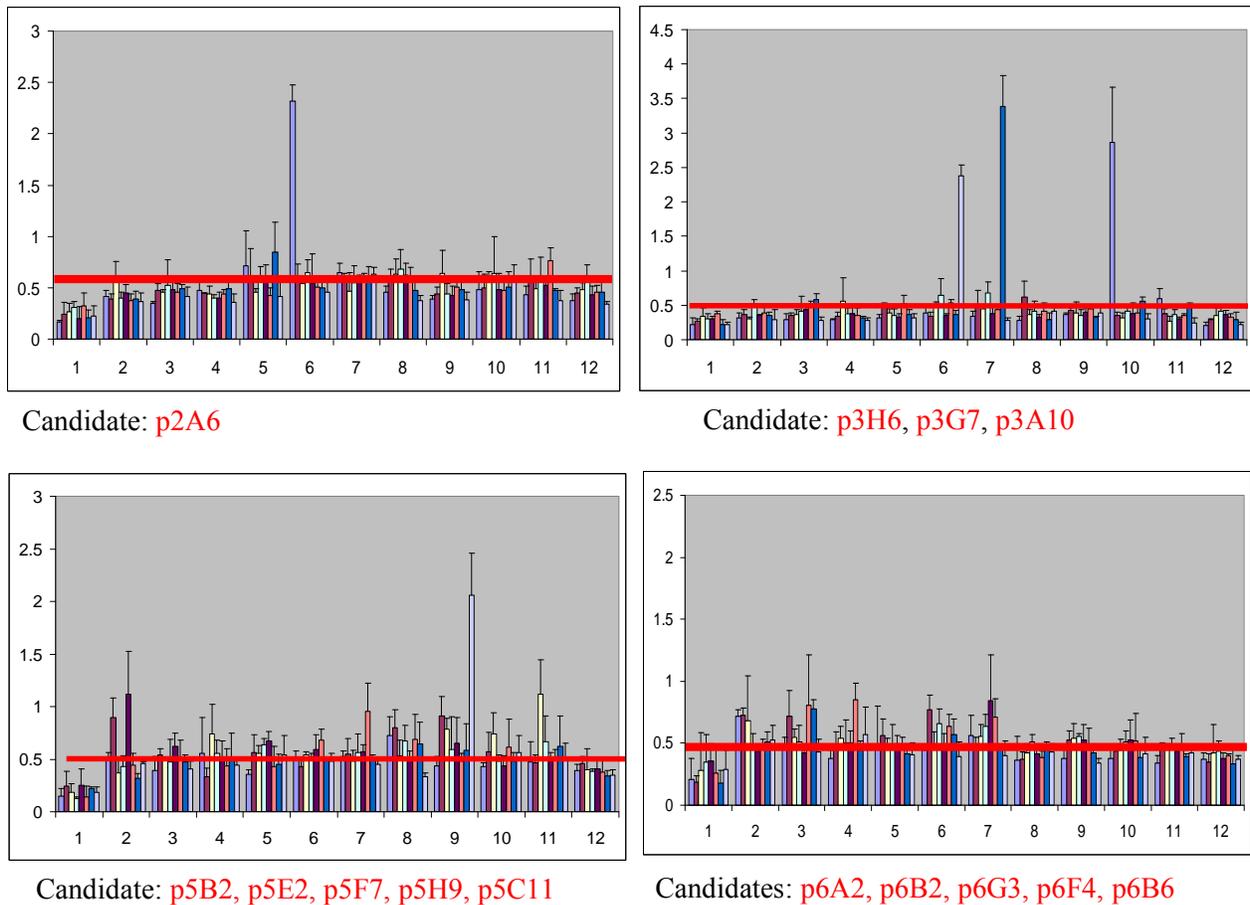
Figure 2. Representation of identification of miR221/222 candidate compounds by using lucifrase assay. "P" = plate; "A-H" = well number.

## 2. Determine the specificity of miR-214 and miR-221/222 inhibitors.

We have previously identifie d 37 candidate com pounds that inhibit miR-214 (see the grant proposal). To determ ine the spec ificity of these com pounds as well as the com pounds that inhibit miR-221/222, we initially examined if the compounds inhibit microRNA processing/biosynthesis (e.g., transcription, Drosha/DGCR8 a nd Dicer com plex). Briefly, afte r treatment of OV2008 and A2780 cells with and without individual candidate compound, the expression levels of 7 representative mature microRNAs (e.g., m iR-19a, miR-33a, miR-34a, miR-138, let-7d, m iR-221 and m iR-214) were examined by qRT-PCR. Unexpectedly, we found th at majority of candidate compounds affect other miRNAs expression. Only 5 m iR-214 and 3 m iR-221/222 candidates had no significant effect on the levels of miRNAs examined. Furthermore, we have tested if these 8 compounds specifically inhibited their targets (e.g., miR-214 and miR-221/222) by examining 1). The expression of target genes of miR-214 and m iR221/222; 2) RIP-ChIP and 3) direct binding of m iR-214 and m iR-221/222 with their corresponding compounds using a m odified procedure of protein-co mpound binding assay (5). Through these procedures, we identified 2 specific inhibitors for miR-214 (Fig. 3-6). We are currentl y

investigating anti-tumor activity of these 2 compounds in cell culture and animal model and are characterizing candidate inhibitors of m iR-19a/b as well as are screening ChemDiv and Chembridge libraries for miR-221/222 inhibitor(s).
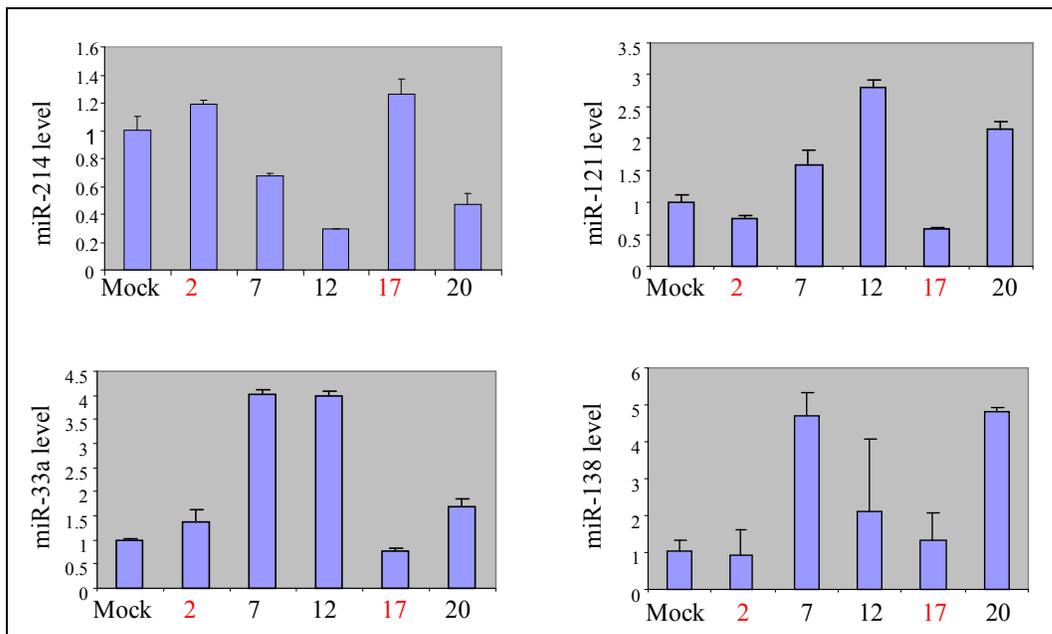
Fig. 3. A2780 cells were treated with each candidate compound or vehicle control (mock) for 12 hours. Total RNAs were isolated and subjected qR T-PCR analysis of 10 m iRNAs. The levels of 4 representative miRNAs were shown. Com pounds #2 and #17 did not significantly affect m iRNA expression.
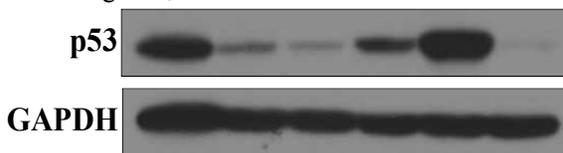

Fig. 4. Compounds #2 and #17 inhibit miR-214-reduced p53 (4) and PTEN (3) expression. A2780 cells were transfected with pre-miR-214 or control oligos. Following 48 h of incubation, the cells were treated with indicated compounds for 12 h and then subjected to immunoblotting analysis with indicated antibodies.
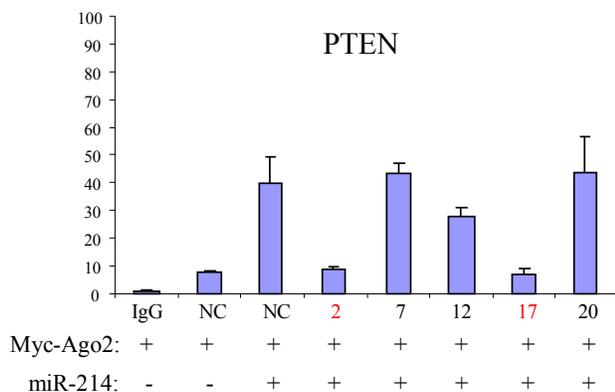
Fig. 5. Compounds #2 and #17 disrupt miR-214 interaction with PTEN in RISC complex. A2780 cells were transfected with myc-Ago2 together with and without pre-miR-214. After 48 h of transfection, cells were lysed. RISC complex was pulled-down by immunoprecipitation with anti-myc antibody and subjected to qRT-PCR for PTEN expression. It is noted that PTEN was enriched in miR-214/RISC complex which was inhibited by compounds #2 and #17.
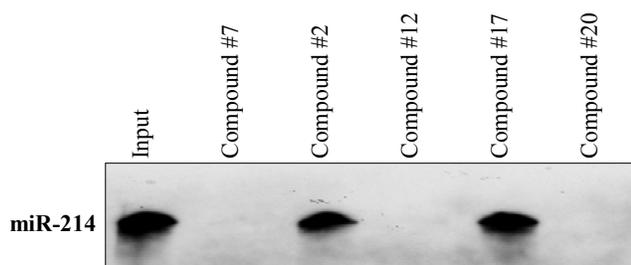
Fig. 6. miR-214 binds to compounds #2 and #17 *in vitro*. The indicated compounds, which contain amino group (5), were immobilized on NHS-activated Sepharose beads and then incubated with *in vitro* transcripted pre-miR-214. After wash and elution, the eluted products were subjected to Northern blot and probed with p32-CTP-labeled miR-214.

Key Research Accomplishment

1. Small molecule compound could directly bind to microRNA.
2. Identification of small molecule inhibitor(s) of miR-214 and miR-221/222.

Reportable Outcomes

Publication:

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Conclusion

Two small molecule compounds could specifically bind to and inhibit miR-214.

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Appendices

MicroRNA MiR-214 Regulates Ovarian Cancer Cell Stemness by Targeting p53/Nanog*[S]

Cheng-Xiong Xu[‡], Meng Xu[‡], Lei Tan[‡], Hua Yang[‡], Jennifer Permuth-Wey[§], Patricia A Kruk[¶], Robert M. Wenham[‖], Santo V. Nicosia[¶‖], Johnathan M. Lancaster[‖], Thomas A. Sellers[§], and Jin Q. Cheng[‡¶1]

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Background: Ovarian cancer stem cells (OCSC) play a critical role in chemoresistance and relapse.
Results: Expression of miR-214 induces, whereas knockdown of miR-214 decreases, OCSC and Nanog. MiR-214 targets p53, a repressor of Nanog.
Conclusion: miR-214 targets p53 to induce OCSC and Nanog.
Significance: MiR-214 is a target for OCSC.

AQ: A
AQ: B
AQ: C

Previous studies have shown aberrant expression of miR-214 in human malignancy. Elevated miR-214 is associated with chemoresistance and metastasis. In this study, we identified miR-214 regulation of ovarian cancer stem cell (OCSC) properties by targeting p53/Nanog. Enforcing expression of miR-214 increases, whereas knockdown of miR-214 decreases, OCSC population and self-renewal as well as the Nanog level preferentially in wild-type p53 cell lines. Furthermore, we found that p53 is directly repressed by miR-214 and that miR-214 regulates Nanog through p53. Expression of p53 abrogated miR-214-induced OCSC properties. These data suggest the critical role of miR-214 in OCSC via regulation of the p53-Nanog axis and miR-214 as a therapeutic target for ovarian cancer.

Fn2

MicroRNAs (miRNAs)[2], small non-coding RNAs that regulate gene expression at the posttranscriptional level, are known to be involved in diverse biological processes, including embryonic development, metabolism, viral infections, and human malignancies. They suppress target gene expression by interaction with complementary sequences in the 3′ UTRs of target mRNAs (1). Certain miRNAs undergo aberrant regulation during carcinogenesis and cause therapeutic resistance and metastasis by regulating multiple target genes. We and others have shown previously that miR-214 is elevated in human malignancy (2–8). In our study of ovarian cancer, up-regulated miR-214 was associated with late-stage and high-grade tumors and induction of cisplatin resistance. In addition, a recent study demonstrated expression of miR-214 promoting migration, invasion, and extravasation *in vitro* and metastatic potential *in vivo* (5). Although chemoresistance and metastasis are charac-

teristics of cancer stem cells (CSC), the role of miR-214 in CSC remains elusive.

Accumulating evidence suggests that CSC are responsible for cancer initiation, progression, metastasis, chemoresistance, and relapse (9). A number of protein-coding genes and pathways regulate CSCs. However, data is emerging to support miR-NAs in CSC regulation (10). For example, recent studies showed differential expression of certain miRNAs between CSC and their differentiated counterparts (10–12), suggesting that miRNA could be involved in the regulation of CSC. In fact, miR-200c and miR-34 have been shown to regulate CSC properties by targeting Bmi1 and down-regulation of Bcl-2 and Notch, respectively (11, 12). Additionally, miR-134, miR-296, and miR-470 modulate embryonic stem cell differentiation by suppression of the expression of transcription factors Nanog, OCT4, and SOX2 (13). Because these transcription factors also play a critical role in CSC (14–16), miRNAs could regulate them to maintain CSC properties (17). In this study, we demonstrate that miR-214 regulates ovarian cancer stem cell (OCSC) property by inducing Nanog through inhibition of p53. Enforcing expression of miR-214 increases, whereas knockdown of miR-214 reduces, OCSC growth preferentially in wild-type p53 ovarian cancer cells. Restoration of p53 largely abrogates the effects of miR-214 on OCSC properties.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, Cell Culture, and Transfection—Expression plasmids of p53 and miR-214 have been previously described (2, 18). pMIR-p53 and MUT-pMIR-p53 were created by ligation of the wild-type and mutant miR-214 binding motif of p53-3′ UTR (746 nt) into the MluI/BamHI sites of pMIR-REPORT vector (Ambion), respectively. Nanog promoter-driven GFP was generated by cloning 806 bp upstream of the Nanog transcriptional starting site, which contains the p53 binding site, into the XhoI/SacI sites of the phrGFP promoter-less vector (Stratagene).

AQ: D

Anti-p53 and -MDM2 antibodies were from Santa Cruz Biotechnology, Inc. Antibodies against BAX, Bmi-1, Sox2, Oct4, Nanog, cleaved PAPR, and cleaved caspase 3 were purchased from Cell Signaling Technology, Inc.

AQ: E

ZSI

[2] The abbreviations used are: miRNA, microRNA; UTR, untranslated region; CSC, cancer stem cell(s); OCSC, ovarian cancer stem cell(s); CDDP, cisplatin; ASO, antisense oligonucleotide.

miR-214 Regulates OCSC via p53/Nanog

The ovarian cancer cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C. Transfections used either siPORT NeoFX transfection agent (Ambion) for oligonucleotides or Lipofectamine 2000 (Invitrogen) for the expression plasmid.

Cell Proliferation and Cell Survival—Cells were plated in 96-well cell culture plates (1×10^4 cells/well) and transfected with indicated oligonucleotides and plasmids. Following incubation for 24 h, cells were treated with and without cisplatin (CDDP, Sigma) or doxorubicin (Sigma) for 24 h. Cell growth and survival were examined with cell counting and an MTT assay according to the instructions of the manufacturer (Sigma). In addition, the programmed cell death was determined by cleavage of PARP and caspase 3.

Quantitative RT-PCR—Total RNA was extracted from cells with TRIzol (Invitrogen) agent according to the instructions of the manufacturer. Reverse transcription and real-time PCR were performed with The TaqMan® MicroRNA reverse transcription kit and TaqMan Universal PCR Master Mix (Applied Biosystems), respectively, using Ambion miRNA primers. The results were calculated and normalized to a control gene (RNU-6B). The mRNA expression levels of p53 and Nanog were measured by semiquantitative RT-PCR. The primer sequences are as follows: p53 (sense), 5′-TTGGATCCATGTTTTGC-CAACTGGCC; p53 (antisense), 5′-TTGAATTCAGGCTC-CCCTTTCTTGCG-3′; Nanog (sense), 5′-ATGCCTGT-GATTTGTGGGCC-3′ and Nanog (antisense), 5′-GCCAGTTGTTTTTCTGCCAC-3′.

Luciferase Assay—Cells were transfected with 0.2 µg of the reporter plasmids, 0.1 µg of pCMV-β-gal, and, where applicable, 5 nm of *miR-214* precursor or control or 50 nm of ASO *miR-214* or control/well on 96-well plates. Following 48 h of incubation, cells were subjected to a luciferase reporter assay using the luciferase assay system (Promega). Luciferase activities were normalized by β-galactosidase activities. Each experiment was repeated at least three times in triplicate.

ALDEFLUOR Assay and Sphere Growth—ALDH1 activity was detected using the ALDEFLUOR assay kit (StemCell Technologies) as described by the manufacturer (19). Briefly, cells were suspended in ALDEFLUOR assay buffer containing an ALDH1 substrate, bodipy-aminoacetaldehyde, at 1.5 µM and incubated for 1 h at 37 °C. A specific inhibitor of ALDH1, diethylaminobenzaldehyde, at a 10-fold molar excess, was used as a negative control. Flow cytometry data were analyzed by BD FACSDiva software V6.1.3 (BD Biosciences) or FlowJo software (TreeStar).

Sphere culture was carried out as described previously (19). Briefly, cells were plated in ultra-negative attachment 6-well plates (Corning) at a density of 5000 viable cells/well. Cells were grown in a serum-free sphere culture medium (MammoCult, StemCell Technologies) supplemented with MammoCult proliferation supplements for 12 days. Sphere numbers were counted under microscopy.

Western Blot and Immunofluorescence—A Western blot analysis was performed as described previously (18). For immunofluorescence, cells were transfected with Nanog promoter-phrGFP together with and without pre-miR-214 or antisense (ASO) miR-214. Following incubation for 48 h, cells were examined with a fluorescence microscope.

Statistic Analysis—Statistical significance was analyzed by unpaired Student's *t* test and one-way analysis of variance and Duncan's multiple range test using SAS statistical software package version 6.12 (SAS Institute, Cary, NC). $p \leq 0.05$ was considered to be statistically significant.

RESULTS

MiR-214 Regulates Ovarian Cancer Stem-like Cells—The findings that miR-214 plays a critical role in chemoresistance and metastasis in ovarian cancer and melanoma (2, 5) prompted us to examine the role of miR-214 in regulating OCSC. Supplemental Fig. S1 shows miR-214 levels and p53 status in a panel of ovarian cancer cell lines and two immortalized ovarian surface epithelial cell lines, T80 and MCC3-HOSE. Because the endogenous miR-214 level is high in A2780S and OV8 and low in OV2008 and SKOV3 cells, we knocked down miR-214 in A2780 and OV8 cells and ectopically expressed miR-214 in OV2008 and SKOV3 cells (Fig. 1*A*). OCSC were detected and isolated by flow cytometry, and sorting of the cells was labeled by ALDH1, a common marker of OCSC (19–21). Fig. 1*B* shows that depletion of miR-214 reduced the ALDH1-positive cell population in wild-type p53 A2780S but not in p53-mutant OV8 cells. Enforcing expression of miR-214 significantly increased OCSC in wild-type p53 OV2008 but not in p53-mutant SKOV3 (Fig. 1*C*). These findings were also confirmed by another OCSC marker, CD133, and in four more cell lines (*e.g.* OV433, OV3, OV10, and A2780CP (supplemental Fig. S2)). Furthermore, sphere growth was evaluated in these cell lines. We observed that knockdown of miR-214 decreased, whereas ectopic expression of miR-214 enhanced, sphere growth in A2780S/OV433 and OV2008/OV10 cells, respectively, with minimal effect on OV8 and SKOV3 cells (Fig. 1*D* and supplemental Fig. S3). Because CSCs closely relate to chemoresistance (22), we further investigated miR-214 effects on chemosensitivity of OCSC. As shown in supplemental Fig. S4, ALDH1-positive A2780S and OV2008 cells expressed high levels of miR-214 and resisted cisplatin and doxorubicin treatment when compared with ALDH1-negative cells. Knockdown of miR-214 in ALDH1-positive cells largely restored their sensitivity to chemotherapeutic agents. These results suggest that miR-214 regulates OCSC in a subset of ovarian cancers.

Nanog Is Regulated by miR-214 in a Subset of Ovarian Cancers—Because accumulating evidence indicates that transcription factors Nanog, Sox-2, and Oct-4 and polycomb ring finger oncogene Bmi-1 are essential to maintain the pluripotent stem cell phenotype (17, 23), we examined the effect of miR-214 on their expression. Western blot analysis and semiquantitative RT-PCR revealed that protein and mRNA levels of Nanog were induced by expression of miR-214 in wild-type p53 OV2008 and OV433 cells (Fig. 2*A*) but not in p53-mutant SKOV3 and OV3 cells (supplemental Fig. S5A). Knockdown of miR-214 decreased Nanog expression in wild-type p53 A2780S and OV10 cells (Fig. 2*B*) but not in p53-mutant OV8 and A2780CP cells (supplemental Fig. S5B). We did not observe significant change of expression in the other three genes examined (Fig. 2 and supplemental Fig. S5).

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miR-214 Regulates OCSC via p53/Nanog

FIGURE 2. **MiR-214 positively regulates Nanog expression in ovarian cancer cell lines expressing wild-type p53.** Indicated cells were transfected with pre-miR-214 (*A*) or antisense of miR-214 (*B*) and control (*Ctr*) oligos. After incubation for 72 h, cells were subjected to Western blot analysis with indicated antibodies (*upper panels*) and semiquantitative RT-PCR analysis (*lower panels*).

Identification of p53 as a Direct Target of miR-214—Because miRNAs negatively regulate their target genes (24), Nanog would not be a direct target of miR-214. Because miR-214 induces Nanog expression at the mRNA level, miR-214 could target a transcription factor(s) that represses Nanog transcription. Previous studies have shown that Nanog is negatively regulated by p53 and positively regulated by Oct4 and Sox2 (15, 25, 26). Our findings that miR-214 regulates OCSC preferentially in wild-type p53 (OV2008, OV433, A2780, and OV10) but not in p53-mutant (SKOV3, OV3, OV8, and A2780CP) cells (27, 28) prompted us to examine whether miR-214 regulates p53. Because OV2008 and OV433 cells express a low level of miR-214, we transfected the cells with pre-miR-214. This resulted in

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with indicated oligonucleotides. After incubation for 72 h, cells were subjected to quantitative RT-PCR analysis of miR-214 expression level (*A*), ALDE-FLUOR (*B* and *C*), and sphere growth (*D*). All experiments were repeated three times (ASO miR214, antisense of miR-214, pre-miR214, pre-miR-214 precursor). *Ctr*, control.   AQ: W

## miR-214 Regulates OCSC via p53/Nanog
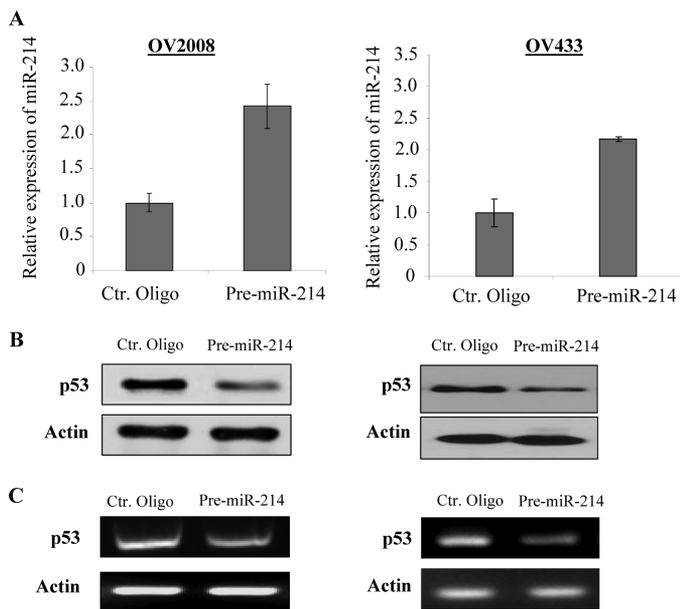
FIGURE 3. **P53 is repressed by miR-214 in wild-type p53 cells.** OV2008 and OV433 cells were transfected with pre-miR-214 or control (*Ctr*) oligo (*A*) and then subjected to immunoblot (*B*) and RT-PCR (*C*) analyses.

FIGURE 4. **Knockdown of miR-214 increases p53 expression in wild-type p53 cells.** A2780 and OV10 cells were treated with antisense (*ASO*) miR-214 or control (*Ctr*) oligo (*A*). After 72 h of incubation, expression of p53 was detected by Western blot analysis (*B*) and semi-quantitative RT-PCR (*C*).

a reduction of about 60–70% of p53 protein and mRNA (Fig. 3, *A–C*). In contrast, A2780 and OV10 cells express a high level of miR-214, so we proceeded to suppress miR-214 expression with miR-214 ASO. This resulted in an increase of p53 expression at protein and mRNA levels (Fig. 4, *A–C*). Furthermore, p53 expression is inversely correlated with miR-214 levels in the majority of ovarian cancer cell lines examined especially in wild-type p53 lines. (Note: SKOV3 is p53-null; supplemental Fig. S6*A*). Immunofluorescence staining showed that the p53 signal was enhanced in A2780S cells transfected with ASO miR-214 but was decreased in OV2008 cells treated with pre-miR-214 (supplemental Fig. S6*B*). In addition, we stably transfected miR-214 into OV433 cells that express wild-type p53 (29) and a low level of miR-214 (supplemental Fig. S1). Immunoblot analysis revealed that the expression levels of p53 and its downstream targets MDM2 and BAX were significantly reduced in miR-214 stably transfected clonal cell lines (supplemental Fig. S7). On the basis of these data, we conclude that miR-214 represses p53.

We next examined whether the 3′ UTR of p53 interacts with miR-214. Because published algorithms (such as PicTar, microRNA.org and DIANA-MICRO) did not show p53 as a potential target of miR-214, we performed a sequence analysis with the RNA22 database and found that the 3′ UTR of p53 contains a region that matches the "seed" sequence of miR-214 (Fig. 5*A*). To ascertain whether miR-214 directly regulates p53, we cloned the 746 bp-3′UTR of p53 containing the wild-type miR-214-p53 response element (MRE), or mutant (MUT) into the pMIR-REPORT plasmid downstream of luciferase. Basal levels of pMIR-p53 reporter activity were examined in two miR-214 low (OV2008 and MCC3-HOSE) and two miR-214 high (A2780 and OV8) cell lines. As shown in Fig. 5*B*, the reporter activity of WT but not mutated (MUT) pMIR-p53 is reversely correlated with miR-214 expression levels. Furthermore, expression of miR-214 represses WT-pMIR-p53 but not
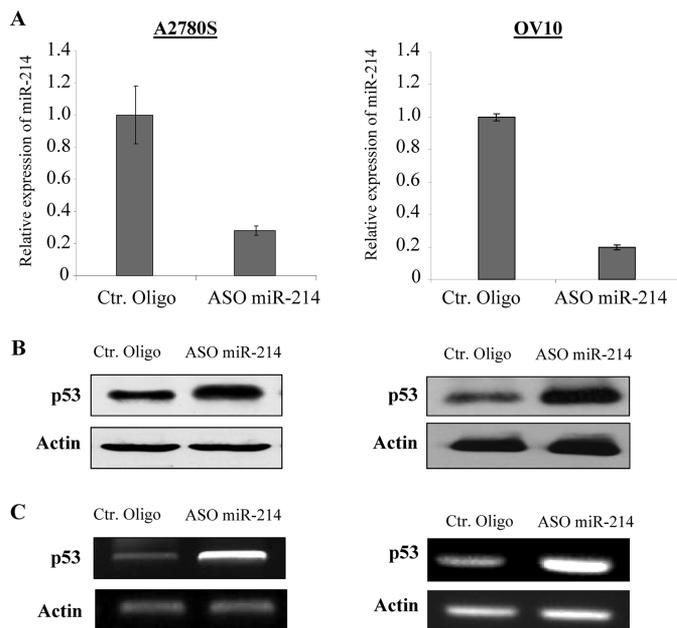
MUT-pMIR-p53 reporter activity in OV2008 (Fig. 5*C*). In contrast, cotransfection of ASO miR-214 and WT-pMIR-p53 in A2780S resulted in an increase of luciferase activity, but the same experiment carried out with the MUT-pMIR-p53 construct resulted in few changes (Fig. 5*D*). As control, expression, and knockdown of miR-199a, an unrelated miRNA, in hTERT-immortalized MCC3-HOSE cells were shown no effects on pMIR-p53 activity (Fig. 5*E*). These data indicate that miR-214 directly targets the p53–3′ UTR at the miR-214-p53 response element to repress p53 expression.

Up-regulation of Nanog by miR-214 through Targeting p53 —Because Nanog is transcriptionally repressed by p53 (25) and is induced by miR-214, we further investigated whether miR-214-induced Nanog is mediated by p53. An 806-bp fragment of the Nanog promoter region, which contains the p53 binding site, was cloned into the pGL3-basic and phrGFP promoter-less vectors (Fig. 6*A*). A luciferase assay revealed that the basal level of Nanog promoter activity is much lower in wild-type p53 OV429 and OV10 than in p53-mutant OV8 and A2780CP cells (Fig. 6*B*). Ectopic expression of miR-214 induced, whereas knockdown of miR-214 decreased, Nanog promoter activity in wild-type p53 but not p53-mutant cells (Fig. 6*C*). Moreover, Nanog promoter-driven GFP expression was increased upon expression of miR-214 and was decreased following miR-214 knockdown in OV429 and OV10 but not OV8 and A2780CP cells (Fig. 6*D*). Accordingly, inverse expression of p53 and Nanog at mRNA and protein levels was detected in miR-214-manipulated OV429 and OV10 cells (Fig. 6*E* and supplemental Fig. S8). In addition, ectopic expression of p53 without the 3′ UTR and knockdown of p53 abrogated the effects of miR-214 on Nanog expression (Fig. 6*F*). Taken together, these data indicate that miR-214 regulates Nanog expression via targeting p53.

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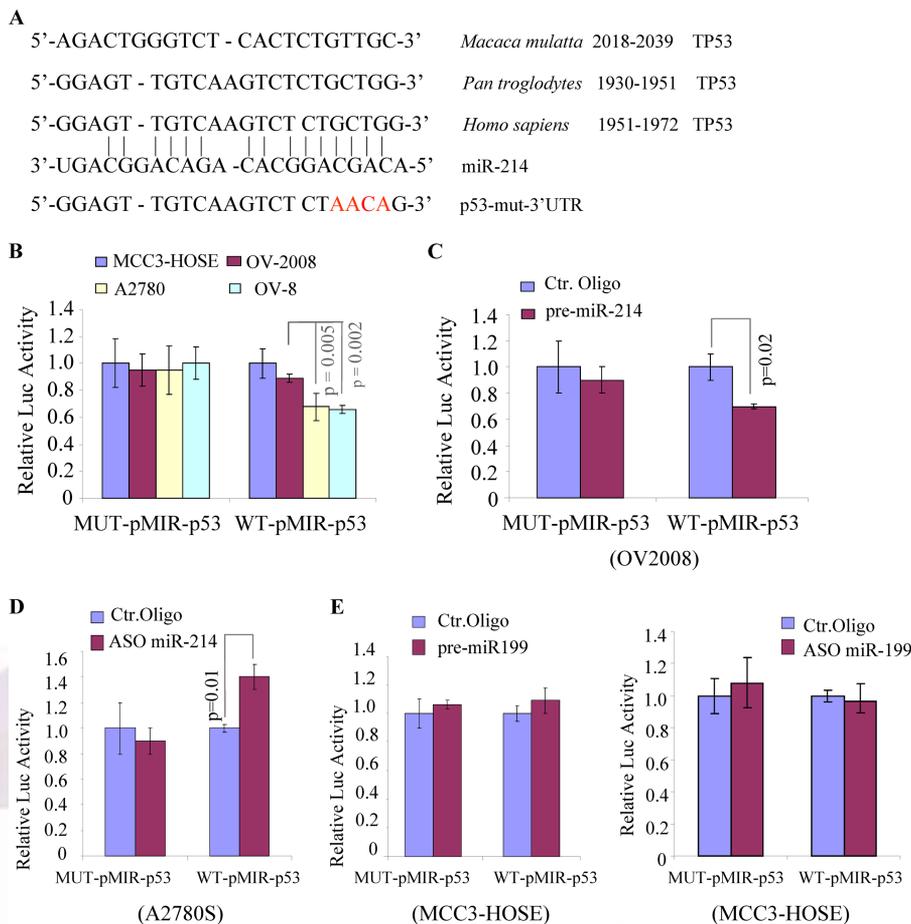
FIGURE 5. **MiR-214 directly interacts with the 3′ UTR of p53.** *A*, sequence alignment of the miR-214 with a 746-bp region of the p53–3′ UTR. *B–E*, indicated cells were transfected with indicated plasmids and oligos. After 48 h of transfection, luciferase activity was measured as described under "Experimental Procedures." The experiment was performed three times in triplicate. *Ctr*, control.

Expression of p53 Overrides miR-214-induced OCSC and Chemoresistance—Having demonstrated that p53 is a direct target of miR-214, we also examined whether expression of p53 will overrule the effect of miR-214 on OCSC property and chemoresistance. Stable miR-214/OV2008 clonal cells were transfected with p53 cDNA without the 3′ UTR. Vector-transfected cells were used as a control (Fig. 7*A*). As shown in Fig. 7*B*, expression of p53 abrogated the miR-214-enhanced OCSC population. Furthermore, expression of miR-214 decreased, whereas knockdown of miR-214 increased, CDDP-induced cleavage of PARP and caspase 3 as well as cell death (Figs. 7, *C* and *D*). However, coexpression of p53 significantly reduced the miR-214 protective effect on cell death induced by CDDP (Fig. 7*E*). These data further support the finding of p53 as a direct target of miR-214 and suggest miR-214 regulation of OCSC and chemosensitivity, at least to some extent, through targeting p53.

DISCUSSION

Up-regulation of miR-214 has been detected in various human malignancies, including pancreatic, prostate, gastric, breast, and ovarian cancers as well as malignant melanoma (2– 8). Furthermore, miR-214 has been shown to play an important role in chemoresistance, tumor progression, and metastasis (2, 5). In this study, we demonstrated that miR-214 regulates ovarian cancer stem cells. Enforcing expression of miR-214 induces, whereas depletion of miR-214 decreases, OCSC properties as well as expression of Nanog. MiR-214 represses p53 by directly interacting with the 3′ UTR of p53. Moreover, we showed that p53 mediates miR-214-induced Nanog, OCSC, and chemoresistance. These findings are important for several reasons. First, they provide a mechanistic understanding of the miR-214 function in OCSC and chemoresistance. Second, these data further support the notion that miR-214 is an oncomiR in human ovarian cancer. Finally, restoration of p53 by inhibition of miR-214 could be a valuable therapeutic approach in ovarian cancer.

Previous studies have shown that miR-214 is deregulated in CSC and high metastatic tumors (11, 30, 31) and that miR-214 induces cell migration and invasion and chemoresistance (2, 5), which are characteristics of CSC (10). These findings implicate an important role of miR-214 in the regulation of CSC. ALDH1 has been proved to be a useful marker for cancer stem cells and has been widely used to isolate CSC in various malignancies, including ovarian cancer (11, 19 –21). In ovarian carcinoma, ALDH1-positive cells exhibit sphere growth, highly tumorigenicity, and resistance to chemotherapy (19, 32–34). Moreover, the expression level of ALDH1 has been correlated recently with a poor prognosis in serous ovarian cancers (19). We

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## miR-214 Regulates OCSC via p53/Nanog
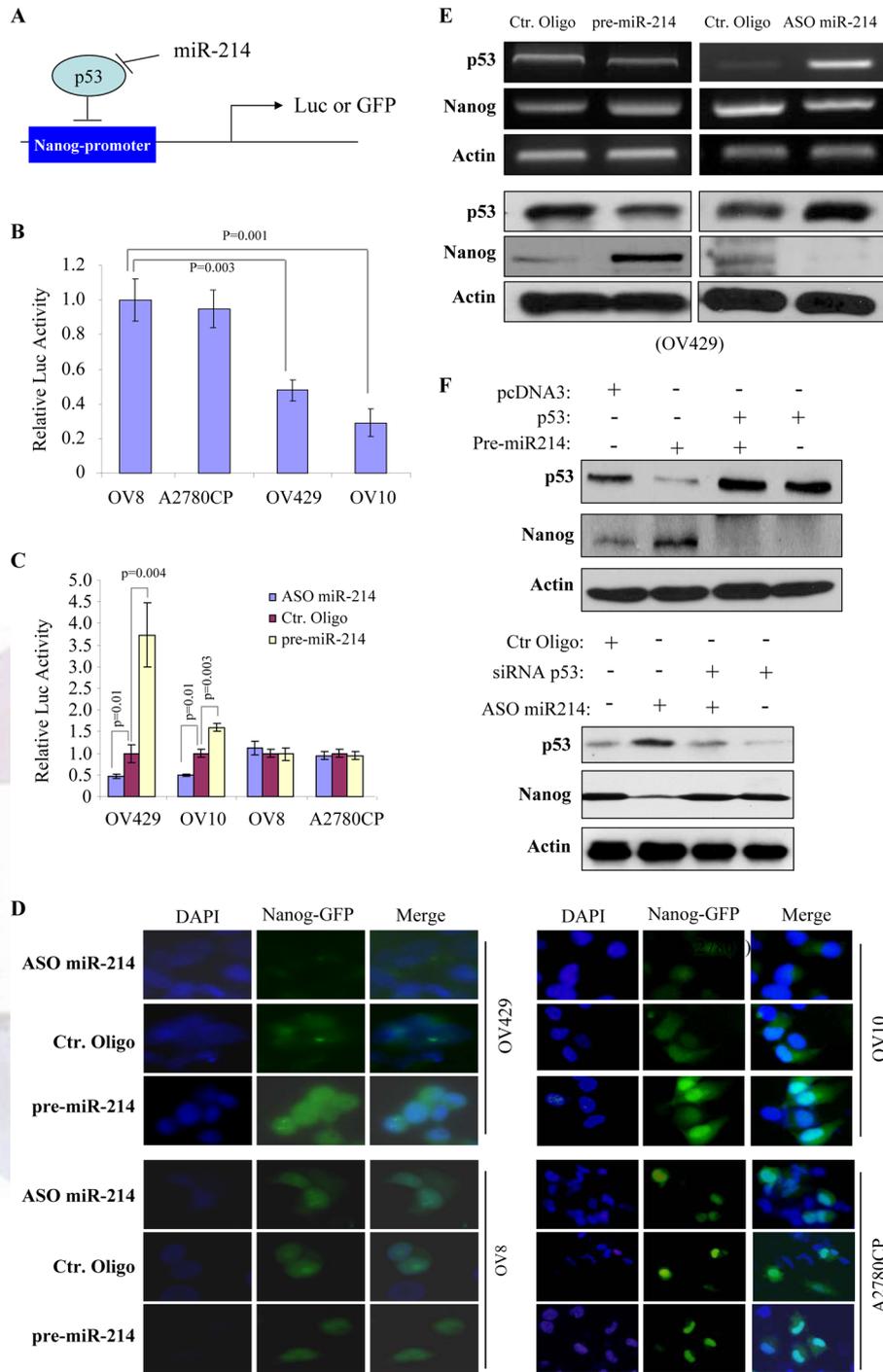
FIGURE 6. **MiR-214 regulates Nanog expression via p53.** *A*, diagram represents an 806-bp fragment of Nanog promoter region was cloned into pGL3-basic or phrGFP promoter-less vectors that are regulated by p53 and miR-214. *Luc*, luciferase. *B*, Nanog promoter activity is lower in wild-type p53 OV429 and OV10 than in p53-mutant OV8 and A2780CP cells. Cells were transfected with pGL3-Nanog-Luc and assayed for luciferase activity after incubation for 48 h. *C* and *D*, expression of miR-214 induces, but knockdown of miR-214 reduces, Nanog promoter activity only in wild-type p53 cells. The cells were cotransfected with pGL3-Nanog-Luc (*C*) or Nanog promoter-driven GFP (*D*) together with indicated oligos. *Ctr*, control. Following incubation for 48 h, cells were subjected to luciferase assay (*C*) and fluorescence imaging (*D*). *E*, p53 expression is repressed by pre-miR-214 and induced by ASO miR-214. OV429 cells were transfected with indicated oligos and then p53 and Nanog levels were examined by RT-PCR (*upper panels*) and immunoblot analysis (*lower panels*) after 72 h of transfection. *F*, expression of p53 abrogates miR-214-induced Nanog, whereas knockdown of p53 inhibits depleting miR-214-repressed Nanog expression. OV433 cells were transfected with pre-miR-214 together with and without p53 (*upper panels*), and OV10 cells were treated with ASO miR-214 in combination with and without siRNA-p53 (*lower panels*). After incubation for 72 h, cells were immunoblotted with the indicated antibodies.

showed in this study that ALDH1-positive ovarian cancer cells express high levels of miR-214 (supplemental Fig. S4*A*) and CSC markers, including LIN28, Nanog, OCT4, and SOX2 (supplemental Fig. S9). Moreover, we demonstrated that knock-

down of miR-214 reduced Nanog expression, ALDH1-positive cell population, OCSC growth, and sensitization of OCSC cells to therapeutic agent-induced apoptosis. In contrast, expression of miR-214 had the opposite effect. Thus, we provide direct
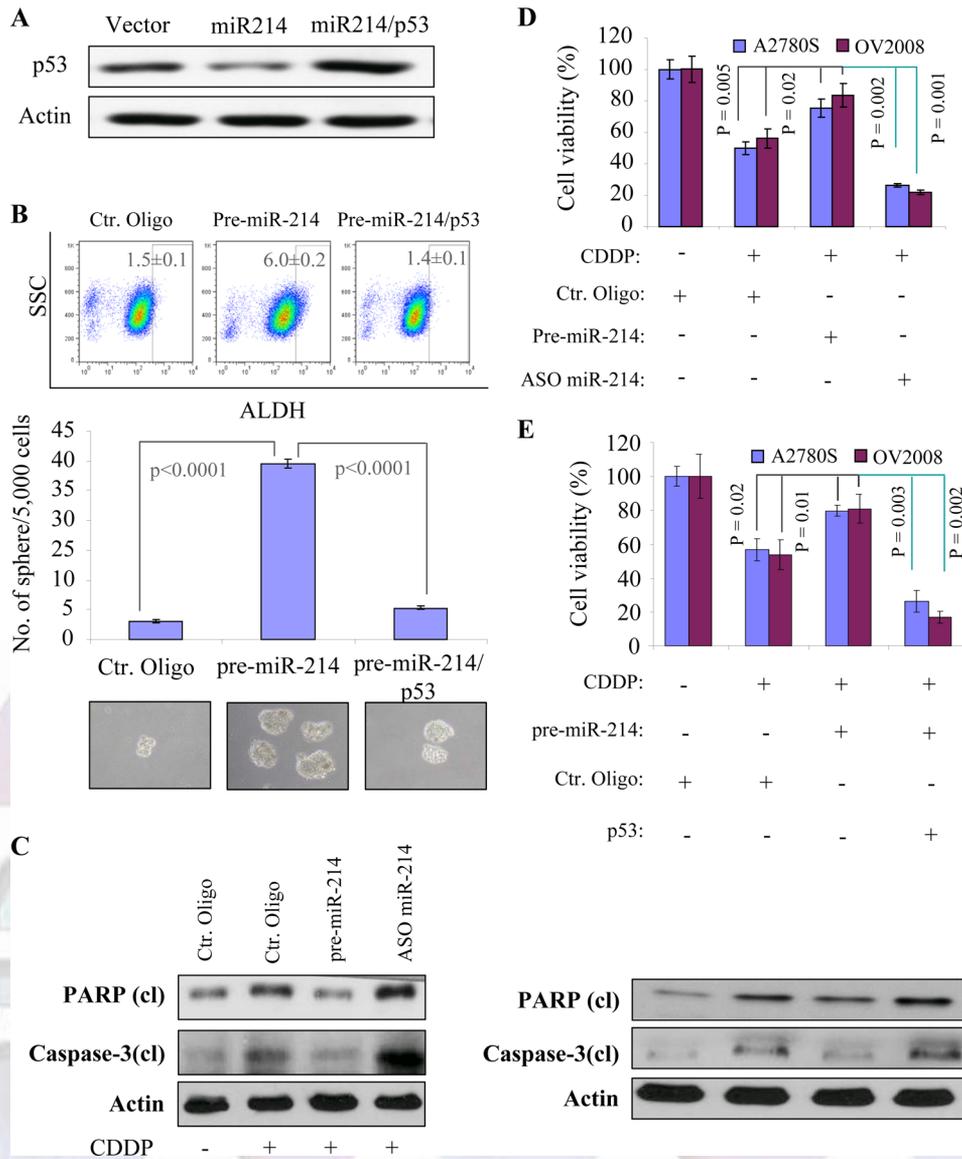
FIGURE 7. Expression of p53 abrogated the effects of miR-214 on OCSC property and chemoresistance. OV2008 cells were transfected with miR-214 together with and without p53 cDNA and then immunoblotted with the indicated antibodies (*A*), ALDEFLUOR, and sphere growth assay (*B*). *Ctr*, control. *C–E*, A2780S and OV2008 cells were transfected with the indicated oligos and plasmid. After 24 h of CDDP treatment, cleaved PARP, cleaved caspase-3, and cell viability were examined. Experiments were repeated three times in triplicate. Note: $p < 0.05$ when comparing the untreated group with the CDDP, CDDP/pre-miR-214, CDDP/ASO miR-214, and CDDP/pre-miR-214/p53 groups. In addition, it is also statistically significant ($p < 0.05$) of CDDP *versus* CDDP/ASO miR-214 and CDDP *versus* CDDP/pre-miR-214/p53.

evidence that miR-214 plays a critical role in maintaining OCSC properties and that miR-214 is a critical therapeutic target in ovarian cancer.

The P53 level, in addition to mutation, has been linked to control of stem cell and CSC properties. Loss of *p53* diminishes spontaneous apoptosis and differentiation of embryonic stem cells. Several studies have shown that loss of *p53* improves the generation of induced pluripotent stem cells from adult cells (35–38). In hematopoietic stem cells, p53 negatively regulates their self-renewal (39). Mice deficient in *p53* show enhanced HSC self-renewal and have an increased HSC pool size. Similarly, loss of p53 has been shown to increase CSC self-renewal, and expression of p53 represses CSC properties (40). Furthermore, p53 has been shown to repress expression of Nanog, a key molecule in maintaining pluripotency and self-renewal of stem

cells, through binding and inhibiting its promoter (25, 41). Because miRNA functions as a regulator of gene expression, it is plausible that miRNA plays an important influence on stem cells through modulation of p53. In fact, a recent study showed that miR-33 enhances HSC transplantation efficiency through down-regulation of p53 (42). We showed in this study that miR-214 represses p53 in ovarian cancer cells expressing wild-type or mutant p53 (Fig. 3 and supplemental Fig. S10). However, miR-214 had no effect on Nanog expression in p53-mutant cells (supplemental Fig. S10) because of the fact that mutated p53 loses its DNA-binding activity (43). Our data showed that miR-214 regulates OCSC primary in wild-type p53 cells even though a number of protein-coding genes such as PTEN, TFAP2C, Sufu, and PTCH are targeted by miR-214 (2, 5, 44, 45). Never-

miR-214 Regulates OCSC via p53/Nanog

theless, these findings further suggest the importance of the miRNA-p53-Nanog axis in regulation of CSC properties.

In summary, we identified p53 as a major target of miR-214. MiR-214 regulates OCSC preferentially in wild-type p53 cells. MiR-214 induces Nanog through p53. Because OCSC has been implicated in ovarian cancer recurrence and relapse, our study suggests that miR-214 could be a pivotal therapeutic target for OCSC, especially in wild-type p53 tumors.

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Supplemental Data

Figure S1. **MiR-214 expression levels and p53 status in ovarian cell lines.** (**A**) qRT-PCR analysis was performed with specific miR-214 primers in indicated cell lines. (**B**) The list of p53 status in ovarian cancer cell lines.

Figure S2. MiR-214 regulates OCSC in wild-type p53 ovarian cancer cells. (**A - C**) Indicated cell lines were transfected with pre-miR-214 or ASO miR-214 and control oligo. After 72 h of transfection, cells were subjected to CD133 labeling (upper panels of A), qRT-PCR analysis of miR-214 expression level (bottom panels of A) and ALDEFLUOR analysis (B, C)

Figure S3. MiR-214 does not affect sphere growth in mutant p53 cells OV8 and SKOV3. Cells were plated in ultra-negative attachment 6-well plate (5,000 viable cells/well) and were grown in a serum-free sphere culture medium for 12 days. Sphere numbers were counted under microscopy.

Figure S4. Knockdown of miR-214 overcomes chemoresistance in ALDH1-positive cells. ALDH1-positive and -negative cells were isolated from A2780S and OV2008 cells by flow sorting and were analyzed for miR-214 levels by qRT-PCR. The ALDH1-positive cells were transfected with antisense of miR-214 (**A**). The cells were treated with or without CDDP (**B**) and doxorubicin (**C**). After 24 h of treatment, cells were subjected to MTT assay. Data shown are mean ± SD for each experimental group and all the experiments were repeated 3 times in triplicate.

Figure S5. MiR-214 does not regulate Nanog in mutant p53 cells. Indicated cells were transfected with pre-miR-214 (**A**) or ASO miR-214 (**B**) as well as control oligo. Following incubation of 72 h, cells were subjected to immunoblot and RT-PCR analyses.

Figure S6. P53 expression was negatively regulated by miR-214. (**A**) qRT-PCR and Western blot analyses show inverse correlation of miR-214 and p53 levels in a majority ovarian cancer cell lines examined. (**B**) OV2008 and A2780 cells were transfected with indicated oligos and then immuno-stained with p53 antibody after 48 h of transfection.

Figure S7. MiR-214 represses the expression of p53 and its downstream targets. OVCA433 cells were infected with lenti-miR-214 and lenti-miR vectors. After selection with puromycin, 3 stable miR-214 clonal cell lines were subjected to immunoblot analysis with indicated antibodies.

Figure S8. Nanog is regulated by miR-214 in wild-type p53 cells. OV10 cells were transfected with indicated oligos and then subjected to RT-PCR (upper panels) and immunoblot analyses (bottom panels).

Figure S9. ALDH-positive cells express CSC markers. ALDH-negative and – positive cells were sorted and then immunoblotted with indicated antibodies.

Figure S10. **MiR-214 represses p53 but does not in duce Nanog expression in p53-mutant cells.** OV3 and A2780CP cells were transfec ted with pre-m iR-214 (left) and ASO miR-214 (right), respectiv ely. After incubation for 72 h, cells were analyzed by immunoblot (upper pan els) and sem i-quantitative RT-PCR (bottom panels) for p5 3 and Nanog expression.

A
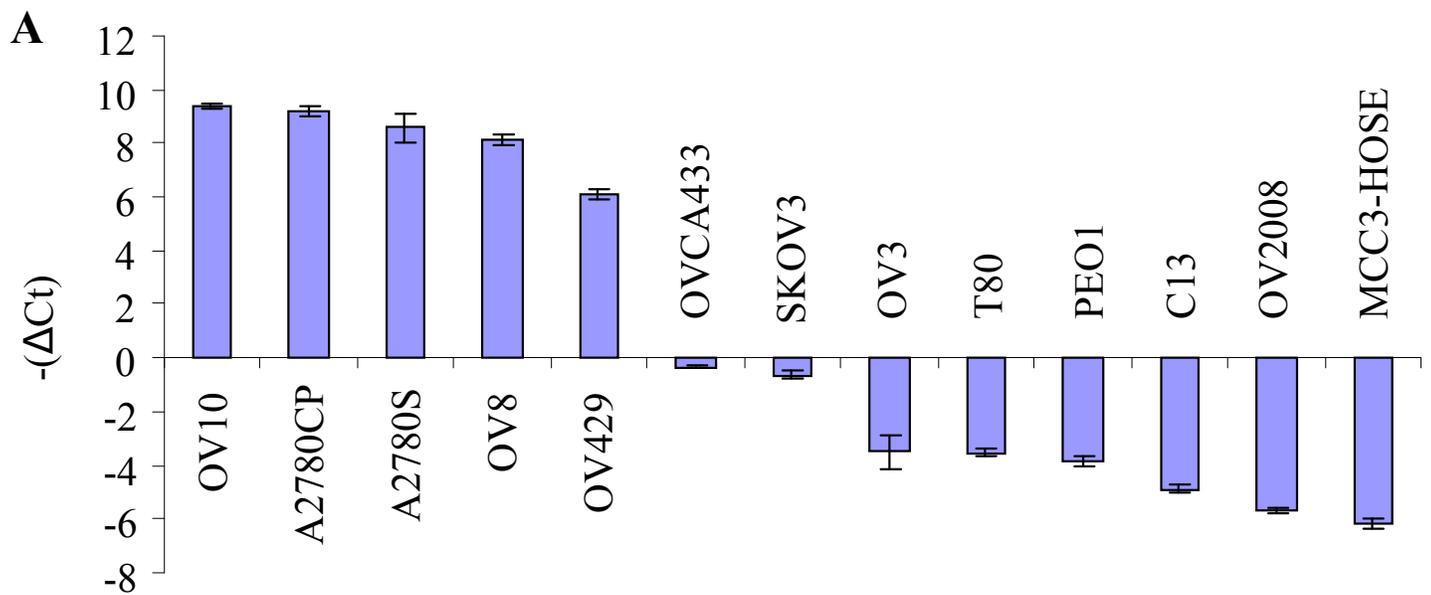
B

Cell line	p53 status
A2780S	Wild type
OV10	Wild type
OV433	Wild type
OV2008	Wild type
OV429	Wild type
A2780CP	Mutation
OV 8	Mutation
OV 3	Mutation
SKOV 3	Null
C13	Wild type
PEO1	Mutation

Figure S1

Figure S2

(OV433)

No. of Spheres/5,000 cells

p=0.02

Ctr.Oligo   pre-miR-214

(OV10)

No. of Spheres/5,000 cells

p=0.04

Ctr. Oligo   ASOmiR-214
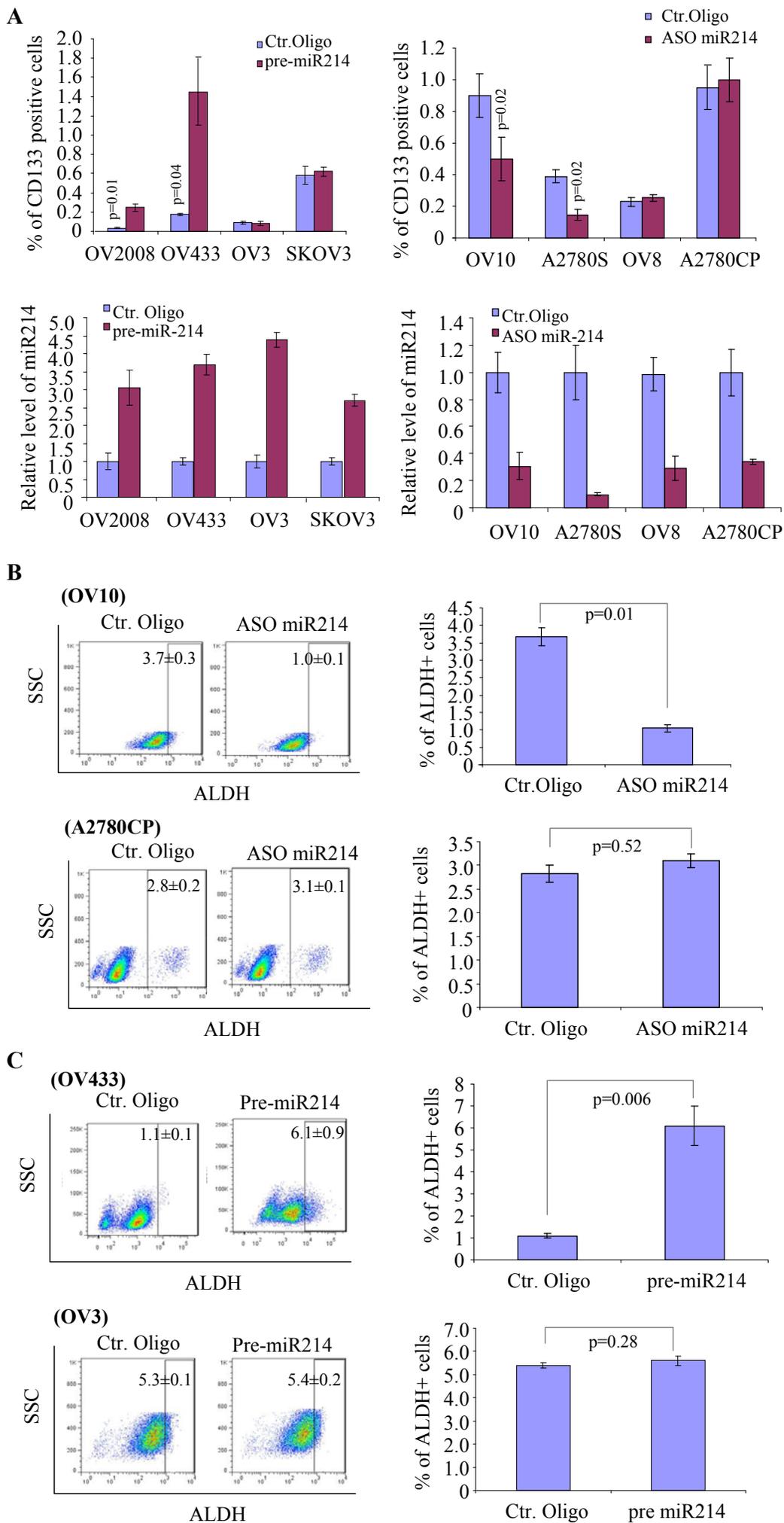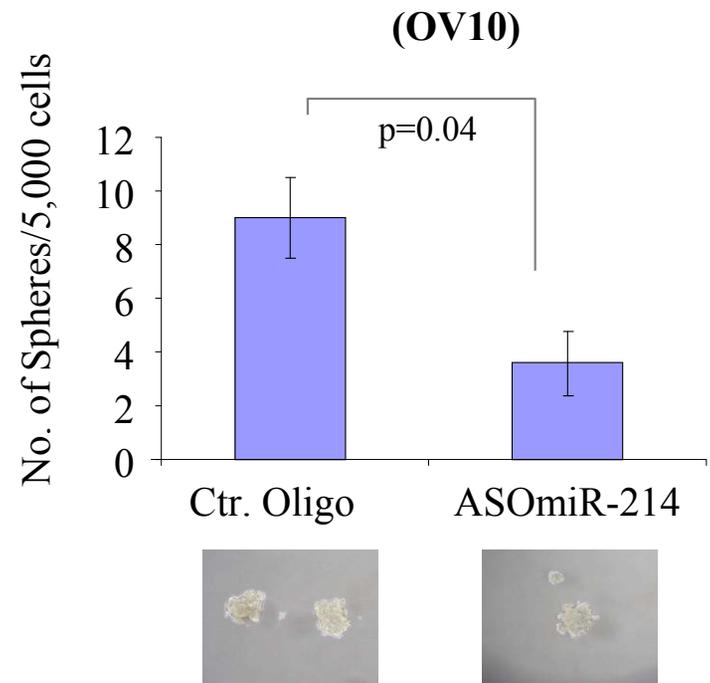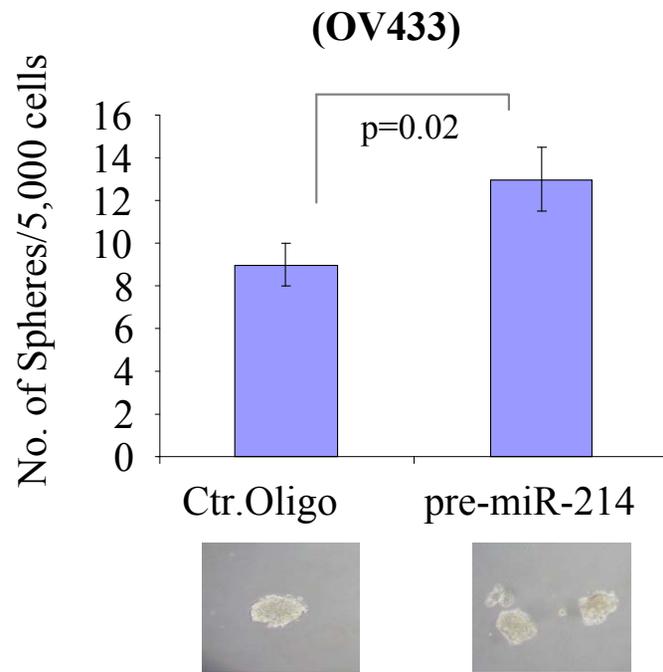
Figure S3

Figure S4

A

B
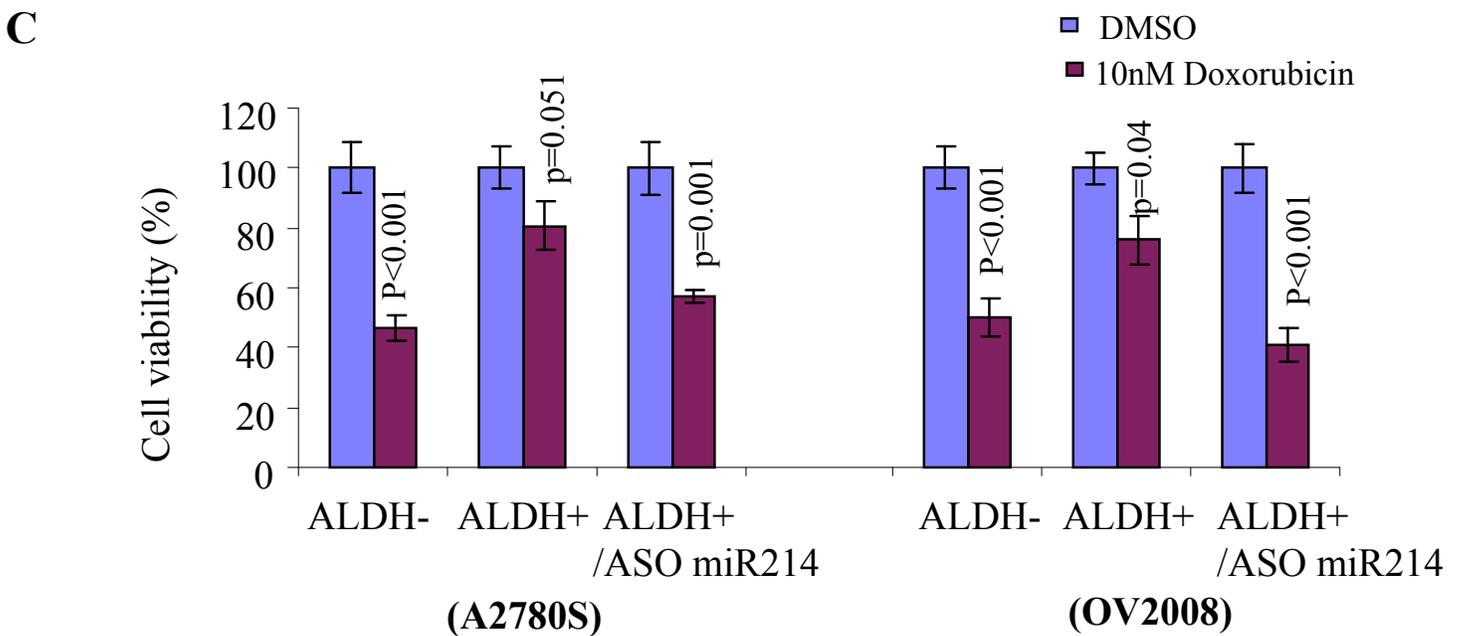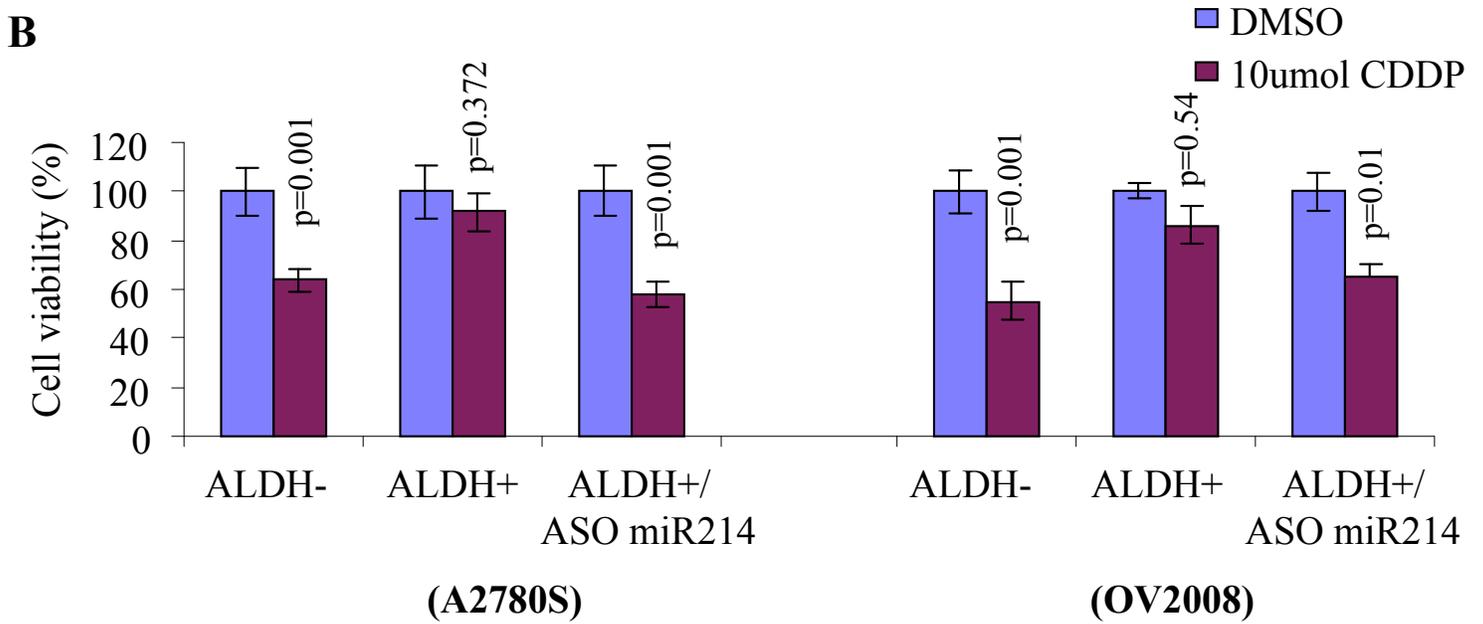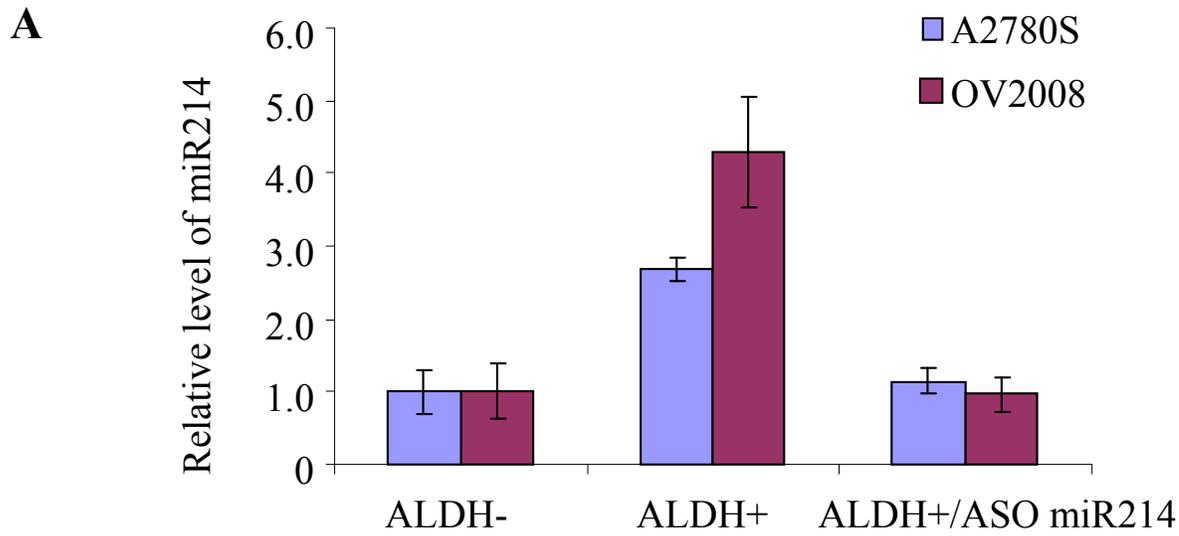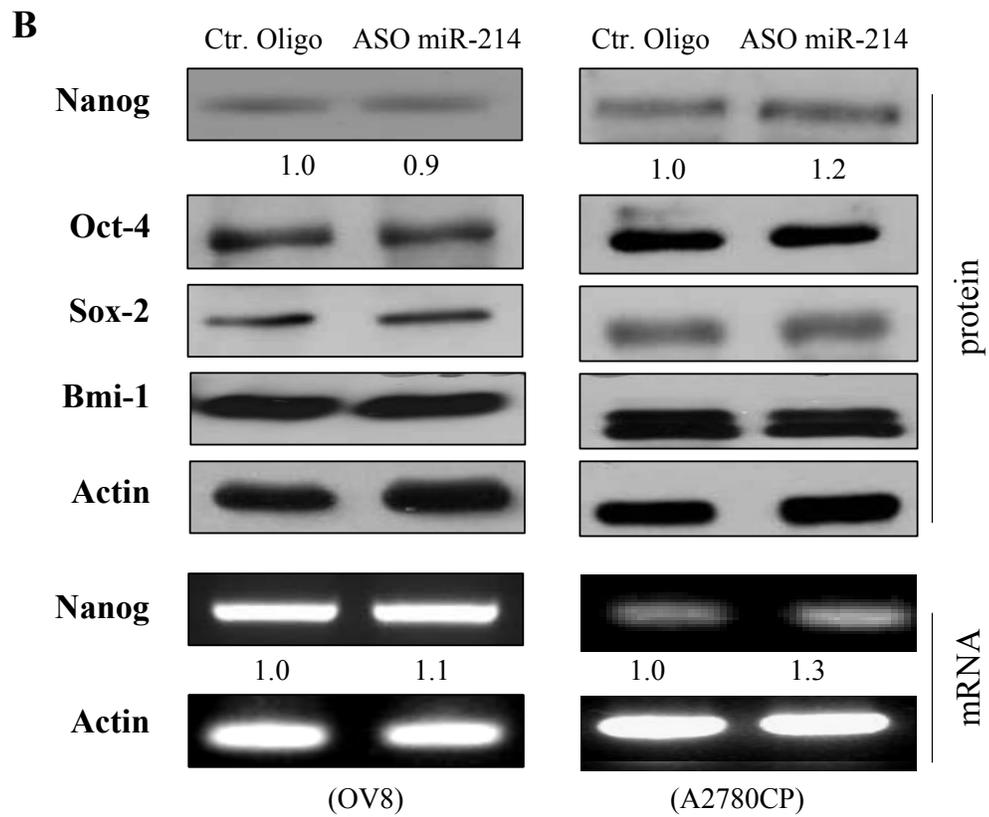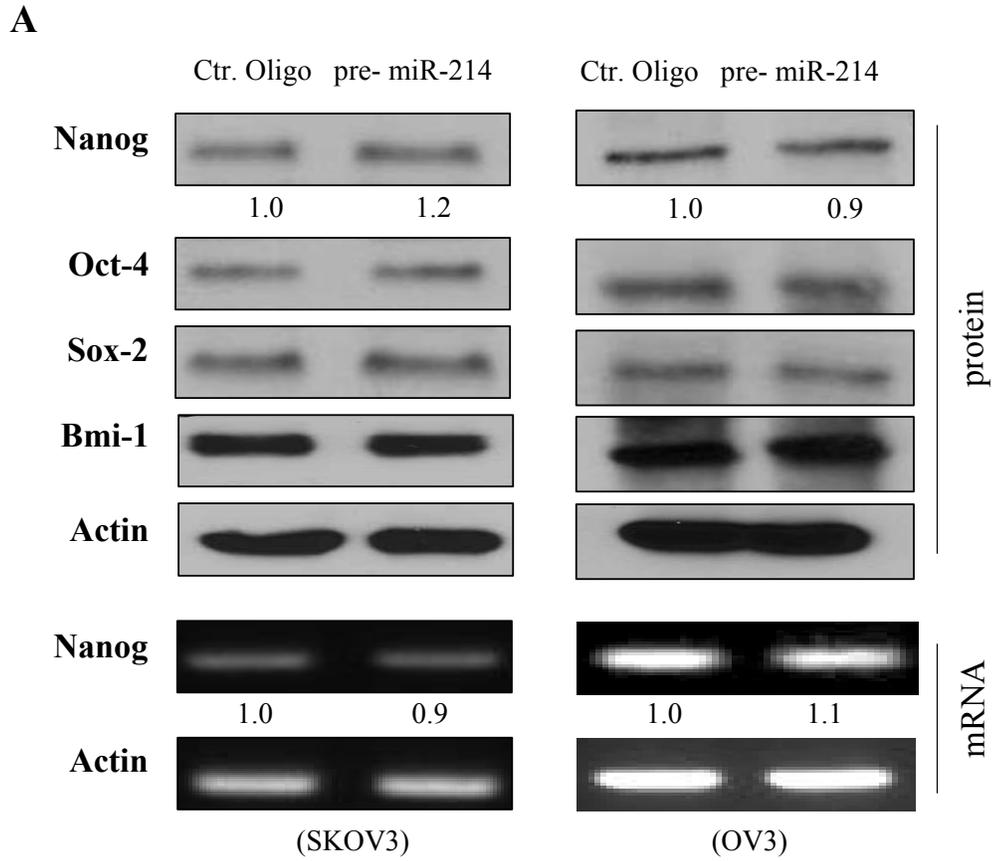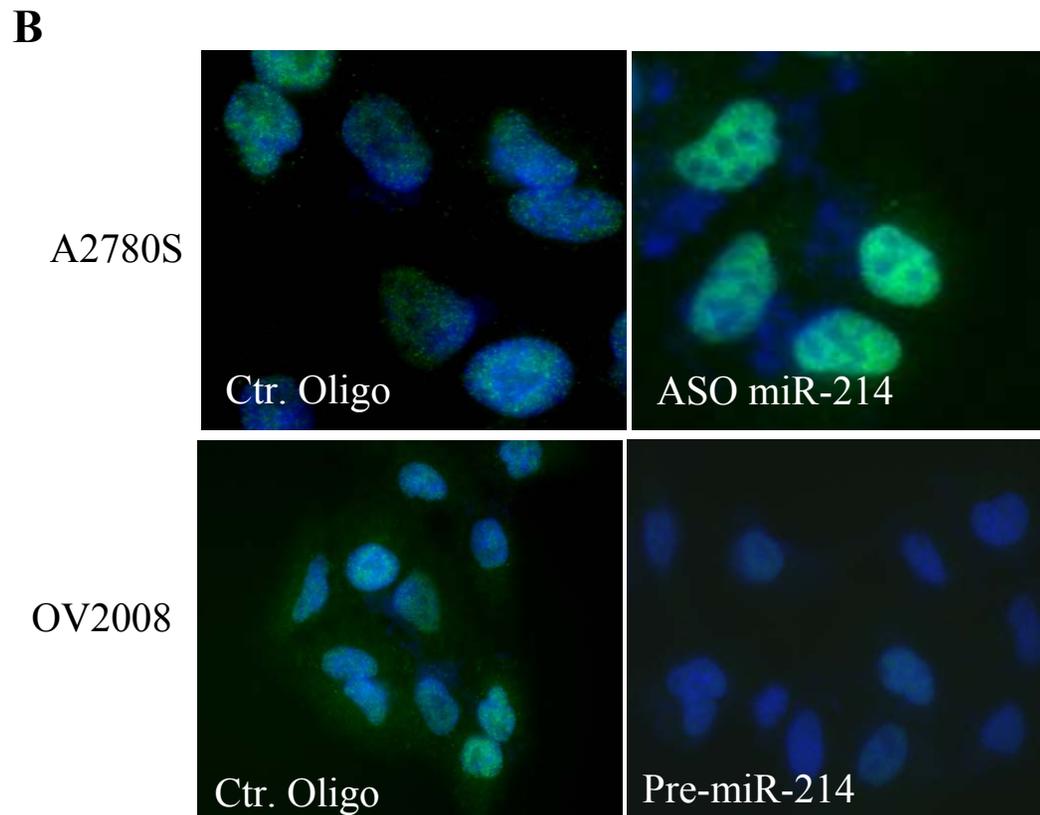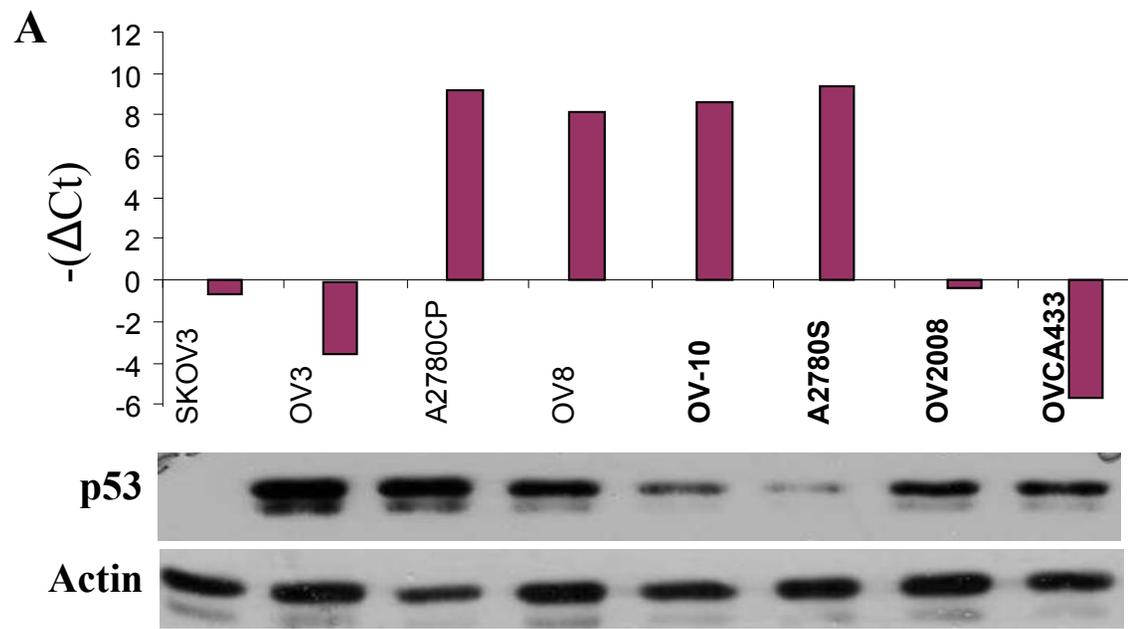
Figure S5

Figure S6

Figure S7

(OV10)

Figure S8

ALDH- ALDH+

Lin28

Nanog

Sox2

Oct4

p53

Actin

(A2780S)

Figure S9

Figure S10